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THE VIABILITY OF *B. TUBERCULOSIS* (*BOVINUS*) ON PASTURE LAND, IN STORED FAECES AND IN LIQUID MANURE.

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I. The viability of *B. tuberculosis* on pasture land.

The object of these experiments was to determine the length of time during which *B. tuberculosis* in cow's faeces remain alive and virulent on pasture land in the south of England. The method of testing for living *B. tuberculosis* is given in Appendix II.

(*a*) *Experiments with naturally infected faeces.*

Great difficulty was experienced in finding the right type of cow for this work, and the faeces of a large number of animals had to be examined before suitable ones were found (Williams and Hoy, 1927). Moreover, it was found that 76 per cent. of the samples from known tuberculous cows gave negative results (Williams and Hoy, 1928). Coupled with the length of time necessarily elapsing before the results of tests were known, this fact caused serious difficulties in the work.

Exp. 1. *Faeces spread naturally.* Cow no. 1 (shown to be excreting *B. tuberculosis* in her faeces) was kept for a few days in a small portable shed without a floor, on pasture land. The cow was then removed and the shed replaced by a wire cage to exclude birds, etc. Monthly samples of the mixture of faeces and churned-up soil from the plot were examined for living *B. tuberculosis*. Six such plots were made at intervals of 1 month. The results were all negative.

Exp. 2. *Faeces spread artificially.* The negative results of Exp. 1 led to the following, in which infected faeces were made up in the form of a cow-dropping and exposed. A preliminary test of a 3-inch layer of faeces had shown some living *B. tuberculosis* after 4 months' exposure (Table I, 1). The following procedure was adopted.

The daily output from the cows was collected, mixed and stored in 5-gallon earthenware jars, which were kept in a hole in the ground and covered with straw. The faeces (of which a sample had shown a positive test for *B. tuberculosis*) were spread on pasture land in patches $2\frac{1}{2}$ to 3 inches thick, each containing 7 to 8 lb., *i.e.* they were of the ordinary dimensions of cow-droppings. Each patch was then covered with a wooden cage 6 feet square and 2 feet high, of which the south side and top were made of wire netting. Five patches were

made. A positive result was obtained from one patch at the end of the first month of exposure, but the five subsequent monthly tests were all negative.

Exp. 3. *Intestinal contents spread artificially.* In this case three patches (23, 25 and 26) were prepared from the intestinal contents of cows which on slaughter had been found to be tuberculous. The initial tests from these patches were positive, but the tests after 2, 3 and 4 months' exposure were all negative.

(*b*) *Experiments with artificially infected faeces.*

The irregularity of positive results in the above series of experiments, due probably to uneven distribution of bacilli in the faeces, led to the following experiments with cow faeces to which known numbers of *B. tuberculosis* derived from tuberculous bovine material (Appendix I) had been added. Results are shown in Table I.

Exp. 4. Patch 7, spread at the end of September, gave a positive result after 4 months' exposure. Patch 8, spread in October, gave a positive result after 3 months, and Patches 9 and 10, exposed in November, gave positive results during 5 monthly tests. Patches 12 (*a* and *b*) were exposed in May 1919 and gave a positive result after 2 months, and subsequently negative results after 4, 5, 6 and 7 months.

Exp. 5. Here the number of *B. tuberculosis* added to the faeces was reduced. Patches 18, 20 and 27 gave an initial positive result, but all subsequent tests were negative. Patch 30, exposed in August 1920, though prepared from the same batch of infective material as Patches 20 and 27, gave a positive result after 4 months' exposure.

The above experiments suggested (*a*) that patches exposed in autumn or winter revealed living, virulent *B. tuberculosis* for longer periods than those set out in spring or summer; (*b*) that the success of the tests for living bacilli was related to the length of time during which faecal material could be observed in the patches. Thus (see Table I, last column) in summer no faecal material was observed after 2 months' exposure, whereas in autumn and winter it might survive 5 months. All attempts to obtain a positive result from the earth after the disappearance of the patches had failed. The following experiment was, therefore, performed.

(*c*) *Experiment with artificially infected faeces exposed under special conditions.*

Exp. 6. Patch 21 was prepared in July 1920 and immediately covered with a large earthenware saucer, supported on bricks, which allowed free access of air and some light, but protected it completely from rain and direct sunlight. This patch is comparable with nos. 20 and 27, but the cover allowed longer survival of faecal material and, correspondingly, of living *B. tuberculosis.*

Faeces exposed on pasture land are subject not only to degradation by heat, air and rain but also to the action of insects and earthworms. To eliminate these factors as far as possible Patch 31 (*a* and *b*) was exposed on a piece of land which had been isolated by a trench filled with coke breeze, and freed as

Table I.

Experiment	Patch no.	Test faeces	No. of bacilli per 20 grm.	Month	Year	Season	At time of exposure	Monthly tests after exposure* 1	2	3	4	5	6	7	Presence of faeces in months
2	2–6	Naturally infected	.	viii	1916	Autumn	25+				+				
			.	ix	1917	„	−	15+	−	−	−	−	−	−	
3	23	Intestinal contents from tuberculous cows	.	vi	1920	Summer	100+		−	−	−				2
	25		.	vii	1920	„	63+		−	−	−				2
	26		.	vii	1920	„	87+		−	−	−				2
4	7	Artificially infected	5,000,000	ix	1918	Autumn	100+	100+	100+	16+	8+	−	−		3½
	8		25,000,000	x	1918	„	100+	100+	100+	100+	−	−	−		3
	9	„	5,000,000	xi	1918	Winter	100+	100+	100+	100+	100+	100+	−	−	5
	10	„	5,000,000	xi	1918	„	100+	100+	100+	100+	100+	100+	−		5
	12 *a*	„	5,000,000	v	1919	Spring	100+		37+		−	−	−	−	
	12 *b*	„	5,000,000	v	1919	„	100+						−	−	
5	18 *a*	„	50,000	v	1920	„	100+		−	−	−				2½
	18 *b*	„	50,000	v	1920	„	100+			−	−	−			3
	20 *a*	„	250,000	vi	1920	Summer	100+		−	−	−				1½
	20 *b*	„	250,000	vi	1920	„	100+			−	−	−			1½
	27 *a*	„	250,000	vii	1920	„	100+		−	−					2
	27 *b*	„	250,000	vii	1920	„	100+			−	−				2½
	30	„	250,000	viii	1920	Autumn	100+		100+	100+	33+	−	−		4½
6	21	(Covered soil)	50,000	vii	1920	Summer	75+		−	66+	16+	−			4½
	22	„	50,000	vii	1920	„	75+	100+	−	−	−				
	31 *a* (Protected from insects, etc.)	„	50,000	viii	1920	Autumn	100+		75+	100+	75+				6
	31 *b* (Protected from insects, etc.)	„	50,000	viii	1920	„						−	8+	−	7

+ =infection of test animals. − =no infection. Blank=no test.

* The numerals preceding the + sign in this and all other tables represent the percentage of inoculations which gave rise to infection. See Appendix II.

far as possible from worm and insect life. As an additional precaution against insects, a hen was allowed access to the ground around the patch which was protected by a wire cage. As a result of these precautions traces of faeces were still visible after 7 months, large numbers of living *B. tuberculosis* were present after 4 months, and they were still demonstrable after 6 months' exposure.

Patch 22 was made from soil only, artificially infected, from the same preparation as Patch 21. Tests after 1 month's exposure gave positive results, but all later tests were negative. This result is included because it yielded a positive result after 1 month's summer exposure, and fills what might otherwise be a gap in the work. To answer fully the question which prompted this experiment, it would be necessary to repeat it with similar soil patches during autumn and winter.

Summary and conclusions.

The results are collected in Table I, and it may be concluded that:

1. *Under ordinary conditions* in the south of England *B. tuberculosis* may remain alive and virulent in cow's faeces exposed on pasture land for at least 5 months during winter, for 2 months during spring and for 4 months during autumn. In summer no living organisms were demonstrated after 2 months.

2. *Under special conditions, e.g.* protection from direct sunlight, the survival period may be 4 months during summer. In autumn faeces protected from earthworms, etc., yielded bacilli after 6 months.

Discussion.

Exp. 6 shows that the length of time during which faecal material remains on the land depends upon the degree to which it is exposed to the influences normally causing its disappearance. It should be noted, too, that the taking of samples from the patches had some effect on the rate of disappearance.

In addition to the seasonal and other influences studied, the conditions in any one piece of pasture may be of importance, *e.g.* a shed or shelter, or overhanging trees which might produce the conditions found in Patch 21 (Exp. 6). The long grass which readily springs up around a cow-dropping may exclude a certain amount of direct sunlight, but at the same time it will keep the site moist and encourage worm and insect activity. It is interesting to note that cows usually avoid this long grass until the faeces have almost disappeared.

The disappearance of organisms from Patch 22 (soil only) after 2 months suggests some action by the soil.

II. The viability of *B. tuberculosis* in stored faeces.

The storage of faeces in the above experiments provided an opportunity of studying the viability of the organism under these conditions.

Exp. 7. About 5 lb. of faeces were allowed to remain in each of the jars, which were covered with loosely fitting lids and stored in a dark cool cellar. Muslin was tied over the lids to exclude insects. The results are shown in Table II.

Table II. *Stored faeces.*

Naturally infected faeces as used for patches.

Year 1917. Month	Test faeces of cow no.	First test	Storage time in months 4	5	6	7	8	9	12	18	24
ii	1	100+	−	25+		−		−			
i	2	100+	25+	100+				33+	12+	−	
	2	100+		50+			−	−	17+	−	
i	2	100+			−		−	50+	−	−	
i	2	−	50+	100+			−			−	

Artificially infected faeces as used for patches.

Patch nos.											
7	100+									8+	
8	100+									87+	66+
9	100+							Surface crust		37+	
								Depth		100+	

+ =infection of test animals.
− =no infection.
Blank=no test.

Conclusion.

Living and virulent *B. tuberculosis* were found after 12 months' storage in the naturally infected faeces, and for a period of at least 2 years in the artificially infected faeces.

III. THE VIABILITY OF *B. TUBERCULOSIS* IN LIQUID MANURE.

Exp. 8. $2\frac{1}{2}$ gallons of liquid manure were taken from a dairy farm storage tank and to each cubic centimetre were added 5000 tubercle bacilli derived from living tissue of tuberculous bovines. The mixture was stored underground in a 5-gallon jar. Before inoculation into test animals the material was concentrated by centrifugalisation and the sediment treated by a modification of Petroff's method (Appendix II).

The results are given in Table III, which shows that living and virulent *B. tuberculosis* were present in the liquid manure after 4 months. There was a gradual falling off in virulence of the material, which becomes more obvious when it is realised that increasing quantities were used for testing as the experiment proceeded.

Table III. *Viability of tubercle bacilli in liquid manure.*

Year 1919. Month	Period of storage (weeks)	Volume (c.c.) of liquid manure per test animal	Result expressed as a percentage of possible infection	No. of guinea-pigs used
v	Nil	50	100+	2
v	1	50	100+	2
vi	4	50	100+	2
vii	6	50	50+	2
vii	8	100	33+	3
viii	10	100	25+	2
viii	10	200	50+	1
viii	12	100	Negative	2
viii	12	200	,,	1
ix	16	200	75+	2
x	22	500	Negative	2

+ =living tubercle bacilli present.

Conclusion.

Living and virulent *B. tuberculosis* were found in stored liquid manure at least 4 months after infection. During this time a gradual diminution of the virulence of the material was observed.

Discussion.

This result is confirmed by those of Brown, Lawrason, S. A. Petroff and F. H. Heise (1916), who found *B. tuberculosis* in sewage contaminated river water "$3\frac{1}{2}$ miles down stream from the outfall"; also those of J. M. Conroy, B. B. Conroy and A. T. Laird (1921 and 1922) who demonstrated *B. tuberculosis* in the effluent from an Imhoff tank at Nopeming Sanatorium. S. L. Cummins, and C. M. Ackland (1929) have recently published a paper in which they give an account of their search for living *B. tuberculosis* derived from "the sludgy film which formed a thin coating over some stones at the point of escape of the effluent from the septic tank." Positive results were obtained by inoculation of the material, after treatment, into guinea-pigs.

APPENDIX I.

PREPARATION OF MATERIAL FOR THE ARTIFICIAL INFECTION OF FAECES.

The badly infected portions were cut out from tuberculous bovine lungs and minced fine by passing twice through a meat mincer. The minced lung was thoroughly mixed with water (about 1000 c.c. to each 2 or 3 lb.) and allowed to stand in a cool place for several days. The mixture was then stirred thoroughly and the presence of *B. tuberculosis* in sufficient numbers was confirmed. It was then allowed to drain in a muslin cloth for several hours. The filtrate was collected, allowed to stand 2–3 days, and any clear fluid syphoned off. The residue was shaken with glass beads for one hour.

The number of bacilli was determined from direct count microscopically, by the method used for milk counting. A good average result was about 5,000,000 bacilli per c.c.

The emulsion of bacilli prepared as above was added to the faeces used for the experimental patches.

APPENDIX II.

METHOD OF TESTING FOR LIVING *B. TUBERCULOSIS* IN THE INFECTED MATERIAL.

The presence of living *B. tuberculosis* was determined by inoculation into two guinea-pigs. The majority of tests were made in triplicate; thus, a total of 60 grm. of material were treated (see below) and six guinea-pigs used. The animals were injected on the inner aspect of each hind leg, making a total of twelve injections.

In some of the earlier tests the faeces and soil were treated with 20 per cent. antiformin before inoculation (occasionally 25 per cent.) in an attempt to reduce local swelling and mortality of the guinea-pigs, caused by resistant organisms other than *B. tuberculosis*. With the introduction of artificially infected faeces it became possible to conduct comparative tests with antiformin and Petroff's method (Petroff, 1915). The results proved conclusively that the antiformin method sometimes gave negative results from material which was shown to contain living bacilli by Petroff's method. The following modification of the latter method was therefore used in all the tests from the artificially infected patches.

Preparation of material for inoculation.

20 to 30 grm. of material, the weight varying according to moisture content, were treated with 150 c.c. sterile water and incubated overnight at 37° C. To the filtrate were added 60 grm. sodium chloride and the scum which floated to the surface on standing was treated with NaOH, neutralised and centrifuged according to the method described by Petroff. The resulting sediment was taken up in distilled water and used for inoculation.

Some idea of the infectivity of the material may be gathered from the percentage (8 per cent. of the total number) of sites inoculated which developed tuberculosis. The positive results in the tables are preceded by a figure which is based on the above. While it leaves much to be desired, it may be taken as a rough guide to the virulence of the material.

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